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Animal 15 (2021) 100347

Contents lists available at ScienceDirect

Animal
The international journal of animal biosciences

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ARTICLE INFO

Article history:
Received 21 November 2020
Revised 28 July 2021
Accepted 30 July 2021

Keywords:
Gene expression
Glucose metabolism
Glycemia
Nutritional programming
Oreochromis niloticus

ABSTRACT

Nutritional programming is a concept proposed to be applied in the field of fish nutrition to improve the use of new diets in aquaculture. This study aimed to investigate for the first time the effects of a glucose injection into the yolk at the alevin stage on intermediary metabolism and growth in adult Nile tilapia (*Oreochromis niloticus*) at 32–37 weeks later in the life. The early stimulus was performed through direct microinjection of 2 M glucose into yolk sacs of Nile tilapia alevin. Subsequently, in adult tilapia, the long-term effects of glucose stimulus on growth performance, blood metabolites, chemical composition in the liver and muscle, expression of genes involved in glucose transport and metabolism (glycolysis and gluconeogenesis) and related pathways (amino acid catabolism and lipogenesis) were investigated. Our results showed that, even though early glucose injection had no effect on growth performance in adult fish, very few significant effects on glucose metabolism were observed. Furthermore, to evaluate the potential metabolic programming after a dietary challenge, a 2 × 2 factorial design with two early stimuli (0.85% NaCl or 2 M glucose) and two different dietary carbohydrate intakes (medium-carbohydrate diet, **CHO-M**; high-carbohydrate diet, **CHO-H**) was performed between weeks 33 and 37. As expected, compared with the CHO-M diet, the CHO-H diet led to decreased growth performance, higher glyceamia and triglyceridemia, higher glycogen and lipid levels in the liver as well as down-regulation of gluconeogenesis and amino acid catabolism gene expressions. More interestingly, although early glucose injection had no significant effect on growth performance, it enhanced the capacities for lipogenesis, glycolysis and gluconeogenesis, particularly in fish that were fed the CHO-H diet. Thus, the nutritional programming of tilapia linked to glucose injection into the yolk of alevins is always visible at the adult stage albeit less intense than what we previously observed in juvenile.

Implications

The effects of early nutritional stimuli in early life may drive permanent changes in physiologic and metabolic pathways in later life. Nile tilapia is able to utilise dietary carbohydrates as the main energy source. Dietary carbohydrate generally provides as the cheap energy source; therefore, the efficient use of carbohydrate would enable least-cost feed. This study demonstrated that hyperglucidic stimulus during alevin stage was associated with modulation of glucose metabolism and its related pathways in long term. The positive effects of hyperglucidic stimulus during larval stage on carbohydrate utilisation in adulthood would facilitate the efficient utilisation of alternative ingredients and/or dietary high carbohydrate for the implementation of cost-effective diets with proper quality.

Introduction

Nutritional programming has been considered to be a possible mechanism for the initiation of human metabolic disorders in adulthood (reviewed in Langley-Evans, 2009; Vickers and Sloboda, 2012). This concept has been extensively studied in different animal models such as rat, mouse and sheep (reviewed in Langley-Evans, 2009; Orozco-Solís et al., 2010; Gopalakrishnan et al., 2005). In general, the effects of early environmental stimuli may drive permanent changes in physiologic and metabolic pathways in later life. Although several mechanisms underlying nutritional programming have been proposed, including changes in

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https://doi.org/10.1016/j.animal.2021.100347
1751-7311/

cell number and cell type, hormonal actions, impaired mitochondrial functions and epigenetics, the precise mechanisms are still poorly understood (for review, see Symonds et al., 2009). In aquaculture, the concept of nutritional programming has been recently applied in fish nutrition research to investigate whether early environmental stimuli can modulate metabolic pathways for better growth and nutrient use in adult fish (for review: Hu et al., 2018; Panserat et al., 2019). This concept was tested using different developmental stages in fish: broodstock, first feeding and embryo-larval stages (Geurden et al., 2007; Rocha et al., 2014, 2015, 2016a; 2016b; Lazzarotto et al., 2015; 2016; Song et al., 2019; Kumkhong et al., 2020a; 2020b).

More precisely, nutritional programming strategies have been successfully used to explore the possibility of modulating some metabolic pathways for a better use of alternative feed ingredients in offsprings through specific broodstock nutrition (Izquierdo et al., 2015; Turkmen et al., 2017; Lazzarotto et al., 2015; 2016). Using zebrafish as a model, parental micronutrient status had long-term effects on differentially expressed genes associated with overall health, lipid utilisation and mitochondrial protein translation in offspring (Skjærven et al., 2016; 2018). In Atlantic salmon, difference in nutritional status between out-of-season and normal spawning season broodstocks influenced their offspring metabolism through nutritional programing (Skjærven et al., 2020). In addition, tests performed at first feeding with a high-carbohydrate diet (**CHO-H**) revealed permanent modifications of carbohydrate metabolism in rainbow trout (*Oncorhynchus mykiss*), nile tilapia and gilthead seabream (*Sparus aurata*), and these findings varied depending on the fish species, stimulus periods, method of stimulus and challenging conditions (Geurden et al., 2007,2014; Rocha et al., 2016a; 2016b; Song et al., 2019; Khumkhong et al., 2020b). Moreover, non-nutritional stimuli such as a hyperglucidic stimulus applied in larvae through direct glucose injection into yolk reserve in zebrafish (*Danio rerio*) have demonstrated the existence of permanent effects of this early stimulus on carbohydrate metabolic pathways in juvenile fish (Rocha et al., 2014 and 2015). Taken together, these data demonstrated the existence of various effects of nutritional programming on metabolism in fish.

Nile tilapia (*Oreochromis niloticus*) is an economically important fish, and its culture is the second largest among freshwater fish after carps (FAO, 2005–2021). Indeed, tilapia nutrition has facilitated the implementation of cost-effective diets with proper quality (Ng and Romano, 2013). Nile tilapia is an omnivorous grazer that can efficiently utilise dietary carbohydrates as the main energy source (Kamalam et al., 2017); therefore, metabolic responses of Nile tilapia to dietary carbohydrates have been intensively investigated (Boonanuntanasarn et al., 2018a; 2018b; Azaza et al., 2015; Wang et al., 2005). In addition, increasing the intake of dietary carbohydrates (more than 40%) is always an objective in this fish species to decrease the feed cost. The positive effects of early stimulus with glucose injection into the yolk to improve dietary carbohydrate utilisation in tilapia juveniles have been recently observed by our laboratory (Kumkhong et al., 2020a). In this study, early injection of glucose (2 M) into the yolk of alevin tilapia, which effectively overloaded glucose content (approximately two times) in yolk reserves, was associated with strong, persistent and positive molecular effects on several carbohydrate-related pathways, including increased glucose transport and glycolysis and decreased gluconeogenesis and amino acid catabolism in juvenile tilapia (Kumkhong et al., 2020a). Moreover, glucose injection during the alevin stage promoted growth and modulated the composition of several plasma metabolites and hepatic and muscle nutrients, suggesting that early glucose injection was linked to highly significant protein sparing effects during the juvenile stage (Kumkhong et al., 2020a). These strong effects of early glucose overload into the yolk reserves modulating several metabolic pathways at the juvenile stage lead to a new question: are these effects of glucose stimulus in alevins could persist through a longer period of life (up to the adult stage)? This is the main objective of the present study.

In this study, effective hyperglucidic stimulus during larval stage was hypothesised to persist through adulthood of tilapia, and to influence carbohydrate metabolism and its related pathways. To deeper study the concept of nutritional programming of carbohydrate in tilapia (which was previously observed up to the juvenile stage (Kumkhong et al., 2020a), we investigated if the effects of early glucose injection into the yolk could be observed up to the adult stage. For this purpose, we evaluated whether the effects of early hyperglucidic stimulus on growth performance and carbohydrate and its related metabolisms could be also detected in adult tilapia (300 g) when the adult fish were challenged with different carbohydrate levels: 67% vs 35%.

Material and methods

Experimental design, experimental fish, microinjection, fish culture and diet formulation

All experimental protocols were approved by the Ethics Committee of Suranaree University of Technology, Animal Care and Use Committee (approval no. A-18/2562). The Nile tilapia broodstock (0.8–1.2 kg) were reared in an earthen pond (800 m^2) at the university farm, Suranaree University of Technology, Nakhon Ratchasima, Thailand. The broodstock were fed with a commercial diet (30% CP, 4% crude fat (**CF**)) at 3% BW at 0900 and 1630 daily. Fig. 1 shows a schematic representation of the experimental design. The experimental design was completely randomised with the injection of two treatment stimuli: 0.85% NaCl and 2 M glucose, each of which included six replicates as previously described (Kumkhong et al., 2020a). The treatment stimuli were conducted by microinjection. For microinjection, fertilised eggs were collected from the mouth of females (six replicates) and reared in a hatching tray (20 × 30 × 5 cm) with flow-through water (27–29 °C) for 3 days. The alevins (stage 17; Fujimura and Okada, 2007) were microinjected with either saline or glucose. The microinjected alevins were reared in the hatching tray with gentle aeration for 1 week. Subsequently, fish (n = 70/replicate) were reared and fed commercial diets (Week 1–4: 40% CP, 5% CF; Week 5–32: 32% CP, 4% CF; Week 25–32: 30% CP, 4% CF) in the cement ponds (2 × 2 × 0.8 m) for 32 weeks.

The combination effects of high-glucose stimulus history and dietary carbohydrate challenge were evaluated during weeks 33–37. A 2 × 2 factorial design with the two stimuli and two dietary carbohydrate levels (medium-carbohydrate diet, **CHO-M**; CHO-H) was employed in a completely randomised design with six replicates (cages). During weeks 33–37, twelve fish were randomly selected from the cement pond and equally distributed into two cages (80 × 90 × 110 cm) to challenge with the challenging diets (CHO-M, CHO-H) (Fig. 1). Table 1 shows the ingredient and chemical composition of challenging diet. The temperature of water (27.0–28.7 °C) and air (30.0–36.0 °C) was determined daily. Dissolved Oxygen (**DO**) and pH were measured weekly, and a DO level of 3.71 ± 0.1 mg L^{-1} and a pH of 7.58 ± 0.2 were considered acceptable. The growth and feed intake parameters were evaluated at the beginning and end of the challenge period and were calculated. Fish mortality was monitored daily throughout the experiment period.

Fish sampling, blood collection and proximate analysis

After week 32 (before challenging), fish were randomly sampled to determine the composition of hepatic and muscle nutrients

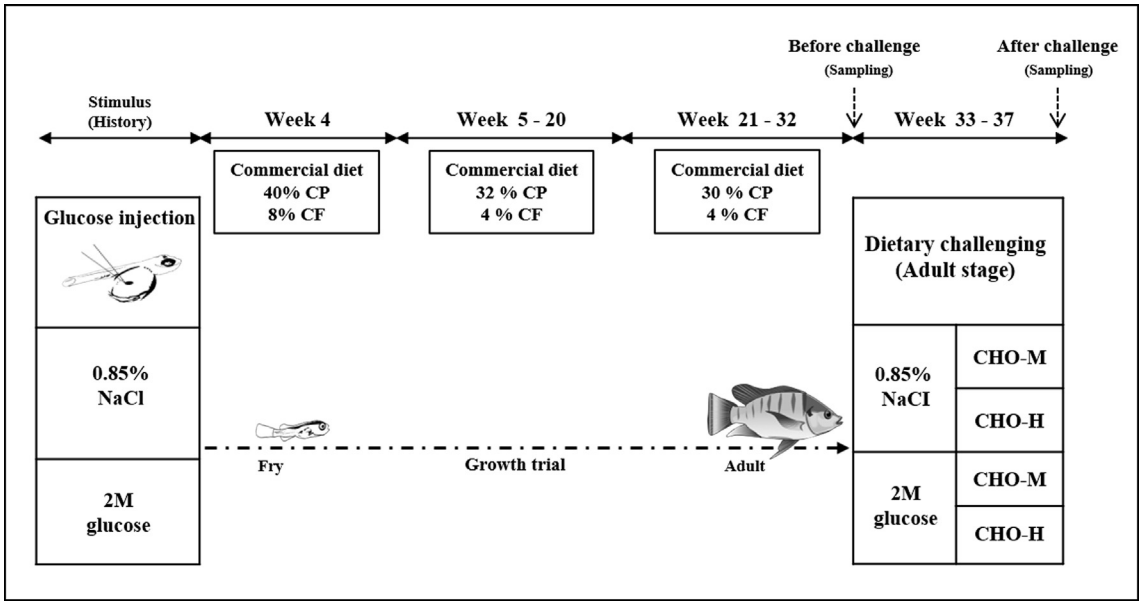

Fig. 1. Schematic representation of the experimental plan of glucose injection stimulus (history) and dietary carbohydrate challenge test (adapted from Kumkhong et al., 2020a). Glucose stimulus was carried out by injecting either a normal saline (0.85% NaCl) or a glucose (2 M) solution into the yolk reserves of alevins. Subsequently, the injected fry were fed with commercial diets according to commercial practices through 32 weeks (adulthood). During weeks 33–37, the adult tilapia was challenged with different dietary carbohydrate levels (37% carbohydrates, CHO-M; 67% carbohydrates, CHO-H). Fish sampling was performed before (week 32) and after (week 37) the challenge test. Abbreviation: CF = crude fat.

Table 1
Ingredients and proximate compositions of the challenging diets for experimental tilapia.

Ingredients	CHO-M	CHO-H
Fish meal	350	140
Soybean meal	300	60
Rice flour	150	700
Rice bran	180	30
Soybean oil	-	40
Di-calcium phosphate	-	10
Fish premix[1]	20	20
Proximate composition (g kg^{-1} dry weight)		
DM	957.7	957.3
Protein	356.7	154.9
Fat	69.0	64.8
Fibre	28.9	8.6
Ash	129.2	60.3
NFE[2]	373.9	668.8
Gross energy (kJ g^{-1})	17.6	17.2

Abbreviations: CHO-M = medium-carbohydrate diet; CHO-H = high-carbohydrate diet.
[1] Vitamin and trace mineral mix provided the following (IU kg^{-1} or g kg^{-1}diet): biotin, 0.25 g; folic acid, 0.003 g; inositol, 0.25 mg; niacin, 0.0215 g; pantothenic acid, 0.03 g; vitamin A, 5 000 IU; vitamin B1, 0.0025 g; vitamin B2, 0.0012 g; vitamin B6, 0.0075 g; vitamin B12, 0.00005 mg; vitamin C, 1 g; vitamin D3, 1 000 IU; vitamin E, 100 IU; vitamin K, 0.008 g; copper, 0.02 g; iron, 0.2 g; selenium, 0.3 mg; zinc, 0.32 g
[2] Nitrogen-free extract = DM − (CP + crude lipid + crude fibre + ash).

(three fish/replication pond). In addition, three fish in each replication pond were randomly selected to analyse blood metabolites, and liver and muscle tissue were collected from two of them to analyse metabolism-related gene expression, enzyme activities and glycogen content. After week 37 (after challenging), the same number of sampling fish was collected from each replication cage.

For sampling, at 5 h after feeding, fish were anaesthetised with clove oil (40 mg L^{-1}). Blood samples were collected from the caudal vein using a hypodermic syringe and transferred into a tube containing 1.0% (v/v) of 15% ethylenediaminetetraacetic acid (**EDTA**). After bleeding, the liver and muscle tissue were dissected and stored at −80 °C for further experiments to analyse gene expression, enzyme activities and glycogen content. The hepatosomatic index (**HSI**) was also determined. Plasma was collected by centrifuging the EDTA blood at 3 000*g* for 10 min at 4 °C and stored at −80 °C until analysis. In addition, to analyse the chemical composition of liver and muscle tissue, fish were anaesthetised with clove oil and then dissected to collect liver and muscle tissue.

Analyses of blood chemicals and chemical composition of liver and muscle tissue

Analyses of plasma metabolites (three fish/replicate), including glucose, triglyceride and blood urea nitrogen (**BUN**), were performed. Plasma glucose levels were determined according to Trinder's method (Trinder, 1969). Triglyceride levels were measured using the glycerol-3 phosphate oxidase-sodium N-ethyl-N-(-3 sulfopropyl)*m*-anisidine method (Bucolo and David, 1973). BUN levels were evaluated using the modified indophenol colorimetric method (Weatherburn, 1967). Proximate chemical analysis, including analyses of protein, lipid and ash, were performed according to the Association of Official Analytical Chemists (AOAC) (1990) methodology. Glycogen composition in liver (100 mg) and muscle (200 mg) was analysed according to a hydrolysis technique described by Good et al. (1933), with modifications (Kumkhong et al., 2020a).

Total RNA extraction and relative quantification of mRNA

Quantitative real-time reverse-transcription polymerase chain reaction (**qRT-PCR**) was conducted for relative quantification of mRNA of carbohydrate metabolism-related genes in liver and muscle tissue (two fish/replication pond or cage; $n = 12$ per experimental group). Total RNA was extracted from liver (50 mg) and muscle (100 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The quantity and quality of total RNA were evaluated using NanoDrop (Thermo Fisher, Madison, WI, USA) and 1% agarose gel

electrophoresis, respectively. One microgram of total RNA was used for cDNA synthesis using a SuperScript III RNAseH-Reverse transcriptase kit (Invitrogen) with random primers (Promega, Charbonniéres, France) following the manufacturer's protocol. Reverse transcription was performed in duplicate for each sample. Table 2 demonstrates the primer sequences used in real-time RT-PCR for the expression of genes that are involved in glucose metabolism, lipogenic capacities and amino acid catabolism in the liver. In addition, the primer sequences used for analysis of real-time RT-PCR of genes which are involved in glucose utilisation and glycolysis in muscle were designed (Table 2). Assays were performed using the conditions described by Kumkhong et al. (2020a). Each PCR assay included replicate samples; therefore, duplicate reverse transcription and PCR amplification assays were conducted to analyse the mRNA level. Reverse transcriptase- and cDNA-template-free samples were used as negative controls. A Roche LightCycler 480 system (Roche Diagnostics, Neuilly-sur-Seine, France) was used for real-time RT-PCR assays. Expression of target genes were relatively quantified using the Roche Applied Science E-Method according to Pfaffl (2001). Because the relative expression of *ef1α* did not significantly vary among the experimental groups (data not shown), it was used as an internal reference for normalisation. A standard curve of each gene expression was created using serial dilutions of cDNA, and PCR efficiency was determined from the slope of the standard curve. In all cases, the PCR efficiency values ranged between 1.8 and 2.0.

Enzyme assays

The hepatic enzyme activities including glocokinase (**GCK**) (EC 2.7.1.2) and pyruvate kinase (**PK**) (EC 2.7.1.40) were analysed using liver (100 mg). In addition, muscle (200 mg) was used to analyse enzyme activities including hexokinase (**HK**) (EC 2.7.1.1) and PK. Tissue samples (two fish/replication) were homogenised in seven volumes of ice-cold buffer (50 mmol L^{-1} Tris, 5 mmol L^{-1} EDTA and 2 mmol L^{-1} DTT; pH 7.4) containing a protease inhibitor cocktail (P2714; Sigma-Aldrich, St. Louis, MO, USA). The homogenates were centrifuged at 900*g* for 10 min at 4 °C, and the subsequent supernatant was performed enzyme assays of GCK and HK. The homogenates were carried out additional centrifugation at 10 000*g* for 20 min at 4 °C. The supernatants were used for the analysis of PK activity. Each enzyme analysis was evaluated in duplicate at 37 °C, and the reactions were started by the addition of a specific substrate. A PowerWave X (BioTek Instruments Winooski, VT, USA) plate reader was used to determine the variation of absorbance of nicotinamide adenine dinucleotide phosphate at 340 nm. The GCK (high-K_M) and HK (low-K_M) assays were measured as described by Panserat et al. (2000). PK activity was also determined as previously described by Panserat et al. (2001). De-ionised water was used as a blank for each sample. Enzyme activity units were calculated as per milligram of protein and defined as micromoles of substrate converted into product per minute at the assay temperature. Protein concentration was analysed in duplicate according to the method of Bradford (1976) using a protein assay kit (Bio-Rad, Munich, Germany) with bovine serum albumin as a standard.

Data analysis

All data were performed statistical analysis using SPSS for Windows, version 12 (SPSS Inc., Chicago, IL, USA). To evaluate differences between the stimuli injection: saline vs glucose, an independent *t*-test was performed. Two-way factorial analysis of variance (ANOVA) was used to analyse the effects of history of glucose stimulus, dietary challenge by high-carbohydrate feeding, and their interactions. One-way ANOVA followed by Tukey's range test was carried out to rank the treatment combination groups. The effects and differences were declared to be significant when *P*-values were less than 0.05 ($P < 0.05$).

Results

Throughout the experimental period (32 weeks), the survival rate was high in both groups that received or did not receive the glucose injection during the alevin stage, ranging from 97% to 99%. During the challenge period (weeks 33–37), there were no significant differences in the survival rate among the experimental groups (Table 3). There was no significant difference in growth performance between the fish injected with glucose and the fish injected with saline at week 32. When both experimental groups were subjected to challenge with different amounts of dietary carbohydrates, no significant effect of glucose stimulus history on growth performance was observed (Table 3). However, the effects of different dietary carbohydrate levels on growth response were observed. As expected, adult tilapia fed with medium-carbohydrate diet (CHO-M) had significantly higher final weight, average daily gain, specific growth rate and feed conversion ratio (Table 3).

The effects of early glucose stimuli history on blood metabolites, including glucose, triglycerides and BUN, are shown in Table 4. There were no significant differences in the levels of these metabolites between the fish injected with glucose and the fish injected with saline at week 32. When these fish were challenged with different amounts of dietary carbohydrates, the effects of glucose stimulus history were detectable for BUN ($P < 0.05$). Fish injected with glucose had lower BUN levels than that of fish injected with saline. As expected, tilapia fed with CHO-H had higher glucose and triglyceride but lower BUN levels than tilapia fed with CHO-M ($P < 0.05$) (Table 4).

Table 5 shows the effects of glucose stimulus history on the composition of chemicals in liver and muscle tissues as well as HSI. At week 32, the composition of protein, fat, ash and glycogen in the liver as well as HSI appeared to be similar between the fish injected with glucose and those injected with saline ($P > 0.05$). However, significant differences in protein and fat contents in the liver were observed when fish were challenged with different amounts of dietary carbohydrate. The fish injected with glucose had higher fat and lower protein contents in the liver than those injected with saline ($P < 0.05$). In addition, as expected, CHO-H diet led to decreased protein content and increased fat and glycogen contents in the liver ($P < 0.05$). Significant interactions were observed in protein and fat contents and HSI, which demonstrated that glucose-injected fish fed with CHO-H had the highest hepatic fat contents and HSI and lowest protein contents (Table 5). Glucose stimulus history significantly affected glycogen content in muscle tissues before week 32 as well as after dietary carbohydrate challenging (week 37) ($P < 0.05$) (Table 5). Moreover, a significant effect of carbohydrate challenge diet was observed in muscle protein content ($P < 0.05$) (Table 5). Indeed, an interaction effect was found which showed that glucose-injected fish fed with CHO-H had the lowest protein contents in muscle tissues ($P < 0.05$).

The long-term effects of early glucose stimuli on the mRNA levels of genes related to glucose metabolism in liver and muscle tissues were demonstrated in adult tilapia at 32 weeks (Table 6). Early glucose stimuli led to decreased mRNA levels of *pfkma* ($P < 0.05$), whereas the mRNA levels of other genes remained unchanged. Indeed, the activities of hepatic GCK and PK as well as muscle HK and PK appeared to be similar between the fish injected with glucose and those injected with saline ($P > 0.05$) (Figs. 2A, C and 3 A, C). At week 37, combination effects of glucose stimulus history and dietary carbohydrate were observed when

Table 2
List of the primers and their sequences used for quantitative real-time reverse-transcription PCR analysis of genes which are involved in carbohydrate and its related metabolism in the liver and muscles of adult tilapia.

Genes	5′/3′ forward primer	5′/3′ reverse primer	NCBI accession number
Reference gene			
ef1[1]	GCACGCTCTGCTGGCCTTT	GCGCTCAATCTTCCATCCC	AB075952
In Liver[2]			
gck	GGGTGGTAGGATTTGGTGTG	TGCTGACACAAGGCATCTTC	XM_003451020
pfklr	GACGAGCGAGTGGAGAAAAC	TGTCTTGATCCGAGGGAATC	XM_003447353
Pklr	AGGTACAGGTCACCCGTCAG	CATGTCGCCAGACTTGAAGA	XM_005472622
g6pca1	AGCGTTAAGGCAACTGGAGA	AAAAGCTAACAAGGCCAGCA	XM_003448671
g6pca2	CTTCTTCCCCCTTTGGTTTC	AGACTCCTGCAGCTCCCATA	XM_013273429
pck1	AAGCTTTTGACTGGCAGCAT	TGCTCAGCCAGTGAGAGAGA	XM_003448375
pck2	TACGTCTTGAGCTCCCGTCT	CCTCCTGGATGATGCAAGTT	XM_019354843
fasn	AACCTGCTTCTCAAGCCAAA	CGTCACCCCTTGTTCTTTGT	XM_013276809
g6pd	GTCACCTCAACCGGGAAGTA	TGGCTGAGGACACCTCTCTT	XM_013275693
asat	GCTTCCTTGGTGACTTGGAA	CCAGGCATCTTTCTCCAGAC	XM_003451918
alat	CACGGTGAAGAAGGTGGAGT	GCAGTTCAGGGTAGGAGCAG	XM_005476466
gdh	CGAGCGAGACTCCAACTACC	TGGCTGTTCTCATGATTTGC	XM_003457465
In Muscle[3]			
glut4	GAGGATGGACATGGAGAGGA	CAGGAAAAGCGAGACTACCG	JN900493
hk1	CGTCGCTTAGTCCCAGACTC	TGACTGTAGCGTCCTTGTGG	XM019360229
hk2	CAGAGGGGAATTCGATTTGA	CCCACTCGACATTGACACAC	XM003448615
pfkma	AGGACCTCCAACCAACTGTG	TTTTCTCCTCCATCCACCAG	XM019349871
pfkmb	TTTGTGCATGAGGGTTACCA	CACCTCCAATCACACACAGG	XM003441476
pkma	TGACTGCTTCCTGGTCTGTG	CAGTGAAAGCTGGCAAATGA	XM005447626

[1] *ef1* = elongation factor (Yang et al., 2013).
[2] Genes involve in glycolysis (glucokinase, *gck*; phosphofructokinase, *pfklr*; pyruvate kinase, *pklr*), gluconeogenesis (glucose-6-phosphatase, g*6pca1* and *g6pca2*; phosphoenolpyruvate carboxykinase cytosolic, *pck1*; mitochondria, *pck2*), lipogenesis (fatty acid synthase, *fasn*; glucose-6-phosphate dehydrogenase, *g6pd*) and amino acid catabolism (glutamate dehydrogenase, *gdh*; alanine aminotransferase, *alat*; aspartate amino transferase, *asat*).
[3] Genes involve in glucose utilisation in muscle (glucose transporter, *glut4*) and muscular glycolysis (hexokinase I/II, *hk1* and *hk2*; phosphofructokinase, *pfkma* and *pfkmb*; pyruvate kinase, *pkma*).

Table 3
Growth performance of tilapia which were injected with either saline (0.85% NaCl) or glucose (2 M) before challenge (week 32) and after (week 37) challenging with CHO-M and CHO-H for 5 weeks (n = 6).

Parameter	Before challenge				After challenge							
	0.85% NaCl History	2 M glucose History	RSE	*P-value*	0.85% NaCl History		2 M glucose History		RSE	*P-value*[1]		
					CHO-M	CHO-H	CHO-M	CHO-H		History	Diet	Interaction
Initial weight (g)	164.3	163.3	1.782	0.949	222.1	223.2	224.6	223.6	2.386	0.141	0.963	0.286
Final weight (g)	222.7	222.6	2.136	0.229	305.8	283.6	308.4	286.4	3.805	0.112	<0.001	0.958
ADG[2] (g day^{-1})	2.3	2.4	0.048	0.257	2.8	2.1	2.8	2.1	0.154	0.990	<0.001	0.949
SGR[3] (% day^{-1})	1.2	1.2	0.025	0.131	1.1	0.8	1.1	0.8	0.056	0.752	<0.001	0.861
FI[4] (g day^{-1})	2.8	2.9	0.040	0.412	2.8	2.5	2.7	2.6	0.301	0.963	0.118	0.389
FCR[5]	1.4	1.4	0.035	0.115	1.0	1.2	1.0	1.3	0.129	0.926	<0.001	0.424
Survival rate (%)	97.3	96.5	6.806	0.111	97.2	94.4	97.2	97.2	7.161	0.646	0.646	0.646

Abbreviations: RSE = residual standard error; CHO-M = medium-carbohydrate diet; CHO-H = high-carbohydrate diet.
[1] Two-way ANOVA was used to analyse the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet).
[2] Average daily gain (ADG) = (final BW − initial BW)/experimental days.
[3] Specific growth rate (SGR) = 100 × [(ln final BW − ln initial BW)/experimental days].
[4] Feed intake (FI) = dry feed fed/experimental days.
[5] Feed conversion ratio (FCR) = dry feed fed/wet weight gain.

Table 4
Blood metabolite of tilapia which were injected with saline (0.85% NaCl) or glucose (2 M) before (week 32) and after (week 37) challenging with CHO-M and CHO-H for 5 weeks (n = 6).

Blood chemistry		Before challenge			After challenge							
	0.85% NaCl History	2 M glucose History	RSE	*P*-value	0.85% NaCl History		2 M glucose History		RSE	*P*-value[1]		
					CHO-M	CHO-H	CHO-M	CHO-H		History	Diet	Interaction
Glucose (mM)	6.3	6.3	0.915	0.866	5.9	6.9	5.6	7.1	0.688	0.876	<0.001	0.254
Triglyceride (mM)	2.5	2.2	0.755	0.562	2.6^{ab}	3.0^{a}	2.1^{b}	3.2^{a}	0.468	0.348	<0.001	0.027
BUN (mM)	0.9	0.9	0.031	0.718	1.1	0.8	0.9	0.8	0.064	<0.001	<0.001	0.219

Abbreviations: RSE = residual standard error; CHO-M = medium-carbohydrate diet; CHO-H = high-carbohydrate diet; BUN = blood urea nitrogen.
[1] Two-way ANOVA was used to determine the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet). Different letters indicate significant differences in the mean values among four combination groups (P < 0.05).

Table 5
Chemical composition and glycogen content in the liver and muscles and hepatosomatic index of tilapia which were injected with saline (0.85% NaCl) and glucose (2 M) before (week 32) and after (week 37) challenging with CHO-M and CHO-H for 5 weeks (n = 6).

Proximate composition (g kg^{-1})	Before challenge				After challenge							
	0.85% NaCl History	2 M glucose History	RSE	*P*-value	0.85% NaCl History		2 M glucose History		RSE	*P*-value[1]		
					CHO-M	CHO-H	CHO-M	CHO-H		History	Diet	Interaction
Liver												
Protein	93.2	94.1	0.239	0.522	126.3^{a}	113.5^{b}	96.3^{c}	93.9^{c}	0.331	<0.001	<0.001	<0.001
Fat	26.2	25.5	0.199	0.593	29.9^{c}	41.3^{b}	29.9^{c}	62.8^{a}	0.624	<0.001	<0.001	<0.001
Ash	11.6	11.5	0.031	0.592	13.3	13.4	13.5	13.5	0.054	0.425	0.715	0.715
Glycogen (mg/g)	126.6	130.3	6.638	0.352	156.2	217.3	149.3	218.5	60.455	0.894	0.006	0.852
HSI (%)	4.2	4.1	0.269	0.367	3.9^{c}	5.2^{b}	3.2^{d}	5.7^{a}	0.388	0.536	<0.001	<0.001
Muscle												
Protein	181.6	183.5	0.425	0.449	183.78^{a}	173.3^{c}	179.7^{ab}	176.1^{bc}	0.357	0.603	<0.001	0.014
Fat	20.0	19.9	0.077	0.799	15.5	14.8	13.5	14.8	0.161	0.136	0.623	0.130
Ash	12.5	12.3	0.012	0.005	13.5	13.5	13.2	13.3	0.061	0.385	0.721	0.871
Glycogen (mg/g)	2.5	3.4	0.583	0.024	2.7	3.7	4.2	5.5	1.649	0.022	0.091	0.783

Abbreviations: RSE = residual standard error; CHO-M = medium-carbohydrate diet; CHO-H = high-carbohydrate diet; HSI = hepatosomatic index.
[1] Two-way ANOVA was used to determine the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet). Different letters indicate significant differences in the mean values among four combination groups (P < 0.05).

Table 6
mRNA levels of genes which are involved in carbohydrate and its related metabolism in the liver and muscles of adult tilapia which were injected with saline (0.85% NaCl) and glucose (2 M) before (week 32) and after (week 37) challenging with CHO-M and CHO-H for 5 weeks (n = 6). Gene descriptions are showed in Table 2.

mRNA level	Before challenge				After challenge							
	0.85% NaCl History	2 M glucose History	RSE	*P*-value	0.85% NaCl History		2 M glucose History		RSE	*P*-value[1]		
					CHO-M	CHO-H	CHO-M	CHO-H		History	Diet	Interaction
Liver glycolysis												
gck	0.6	2.4	1.675	0.115	0.7	1.0	1.3	1.7	0.684	0.039	0.233	0.890
pfklr	1.0	0.9	0.387	0.722	1.1	0.8	1.2	0.8	0.426	0.872	0.059	0.923
pklr	1.2	0.8	0.696	0.282	0.8	1.0	0.8	0.9	0.564	0.903	0.649	0.925
Liver gluconeogenesis												
g6pca1	1.9	0.9	0.982	0.102	0.7	0.8	1.3	1.1	0.505	0.042	0.721	0.555
g6pca2	1.5	0.5	0.889	0.100	0.8^{b}	0.6^{b}	1.7^{a}	0.6^{b}	0.533	0.064	0.003	0.044
pck1	2.0	0.3	1.222	0.063	0.3^{ab}	0.1^{b}	0.7^{a}	nd	0.350	0.200	0.003	0.116
pck2	1.1	0.8	0.381	0.185	0.9	0.4	1.2	0.5	0.652	0.567	0.035	0.622
Liver lipogenesis												
fasn	1.0	0.9	0.560	0.775	1.0^{ab}	0.6^{b}	0.6^{b}	1.7^{a}	0.750	0.231	0.202	0.011
g6pd	0.9	0.9	0.460	0.951	0.8	1.0	1.4	0.9	0.680	0.432	0.625	0.191
Liver acid catabolism												
asat	1.7	0.9	0.813	0.172	0.7	0.4	1.6	0.5	0.605	0.049	0.004	0.109
alat	1.2	1.1	0.509	0.880	0.7^{b}	0.9^{ab}	1.3^{a}	0.8^{ab}	0.353	0.100	0.156	0.034
gdh	1.3	1.0	0.678	0.548	1.0	1.0	1.0	1.0	0.280	0.855	0.866	0.989
Glucose transport and muscle metabolism												
glut4	0.9	0.9	0.412	0.984	0.3	1.3	1.0	0.8	0.766	0.625	0.166	0.037
hk1	1.1	1.0	0.413	0.929	1.1	0.9	1.0	0.8	0.529	0.688	0.388	0.852
hk2	1.0	1.1	0.381	0.935	0.8	0.8	1.0	1.2	0.340	0.046	0.330	0.597
pfkma	1.3	0.3	0.640	0.032	1.4	0.4	0.6	0.7	0.802	0.592	0.213	0.048
pfkmb	0.9	1.1	0.183	0.270	1.0	0.8	0.9	0.7	0.331	0.678	0.088	0.962
pkma	0.9	0.9	0.213	0.591	1.7^{a}	1.0^{ab}	0.6^{b}	0.8^{b}	0.553	0.006	0.199	0.033

Abbreviations: RSE = residual standard error; CHO-M = medium-carbohydrate diet; CHO-H = high-carbohydrate diet.
[1] Two-way ANOVA was used to determine the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet). Different letters indicate significant differences in the mean values among four combination groups (P < 0.05).

fish were challenged with the CHO-H diet (Table 6). The results showed that early glucose stimuli led to elevated hepatic *gck*, *g6pca1*, *asat*, *hk2* and decreased *pkma* mRNA levels (P < 0.05) (Table 6), whereas the mRNA levels of other carbohydrate metabolism-related genes (*pfklr*, *pklr*, *g6pca2*, *pck1*, *pck2*, *fasn*, *g6pd*, *alat*, *gdh*, *glut4*, *hk1*, *pfkma*, *pfkmb*) remained unchanged (P > 0.05). In addition, the enzyme activities of *gck* and *hk* were up-regulated following glucose stimulus (P < 0.05) (Figs. 2B and 3B). Significant effects of the amount of dietary carbohydrate were also observed in several genes involved in gluconeogenesis and amino acid catabolism. Expectedly, CHO-H led to down-regulation of *g6pca2*, *pck1*, *pck2* and *asat* (P < 0.05) (Table 6). Interactive effects between glucose stimuli and dietary carbohydrates were observed for the expression of *g6pca2*, *fasn*, *alat*, *glut4*, *pfkma* and *pkma* as well as hepatic and muscle PK activities (P < 0.05). Based on the interaction effects, the highest up-regulation of *fasn* and hepatic and muscle PK enzyme activities were observed in glucose-injected fish fed with CHO-H, whereas the highest *alat* mRNA level was observed in glucose-injected fish fed with CHO-M (P < 0.05) (Table 6, Figs. 2D, and 3D).

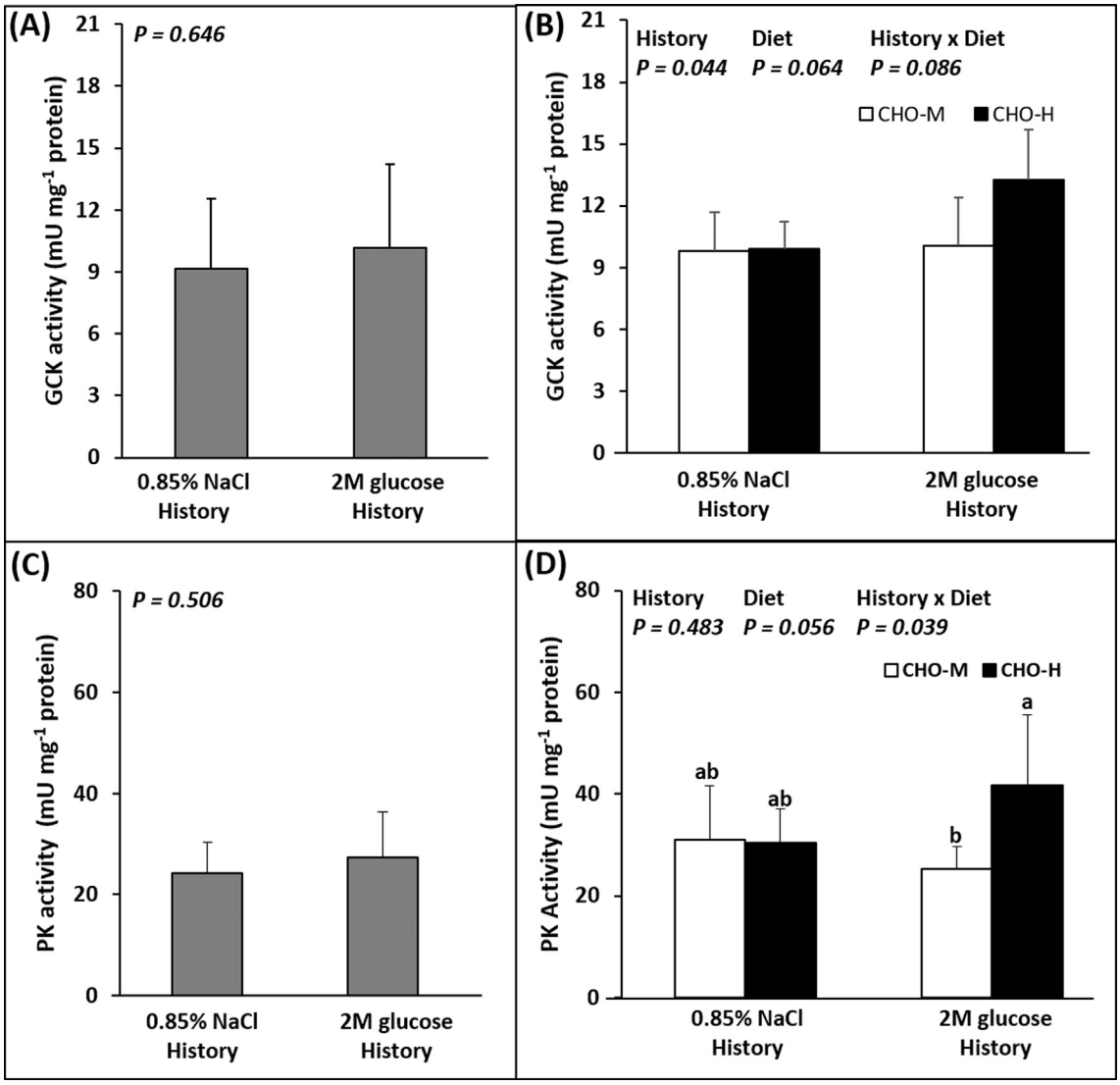

Fig. 2. Enzyme activity (mU mg^{-1} protein) in liver of adult tilapia that were injected with either 0.85% NaCl or 2 M glucose (history). Enzymes involved in hepatic glycolysis included glucokinase (GCK) (A, B) and pyruvate kinase (PK) (C, D) in liver. During weeks 33–37, fish were subjected to challenge with different dietary carbohydrate diets (37% carbohydrates, CHO-M; 67% carbohydrates, CHO-H). Enzymes were analysed to determine the effect of glucose stimuli on enzyme activities at week 32 (A, C) and the combination effects of glucose injection history and dietary challenging at week 37 (B, D). Data are presented as the mean ± SD (n = 6). An independent t-test was used to analyse the effects of glucose injection. Two-way ANOVA was conducted to determine the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet). One-way ANOVA followed by Tukey's range test was performed to rank the treatment combination groups when significant interaction effects were observed. Different letters in the bar graph indicate significant differences ($P < 0.05$).

Discussion

Nutritional programming concept in fish nutrition is still under investigations for tailoring fish metabolism. Glucose injection into yolk reserve is a direct method for fish larvae to experience a high-glucose stimulus. Recently, direct glucose injection into yolk reserve was shown to be effective for permanent modification of glucose metabolism in juvenile zebrafish and juvenile Nile tilapia (Rocha et al., 2015 and 2016; Kumkhong et al., 2020a). Kumkhong et al. (2020a) assessed successful nutritional programming up to the juvenile stage. It is interesting to explore whether the effects observed in juveniles will be always observed up to the adulthood, the programming mechanism may be faded with time. The present study provides novel information in adult Nile tilapia by evaluating the effects of direct high-glucose injection into yolk reserve on growth performance, blood metabolite levels (glucose, triglyceride and BUN), liver and muscle tissue compositions as well as glucose metabolism.

Glucose injection has no long-term effects on the growth performance and postprandial plasma metabolite profiles in adult Nile tilapia

The present results showed no significant effects of glucose stimulus history on growth performance in adult tilapia (up to week 33). In agreement, glucose injection into the yolk sac (2.2 M) in zebrafish embryo during 30% epiboly stage did not change the growth performance during the juvenile stage (Rocha et al., 2014, 2015). This is in contrast to positive effects of early glucose injection on growth performance in fingerling/juvenile tilapia at 16 weeks of age (Kumkhong et al., 2020a). Regarding dietary challenge with the CHO-M and CHO-H diets (after week 33), as expected, a medium-carbohydrate (37%)/medium-protein (35%) diet led to a better growth performance in adult tilapia than that in fish fed with a high-carbohydrate (67%)/low protein (15%) diet, as observed previously (Boonanuntanasarn et al., 2018a; 2018b; Kumkhong et al., 2020a; 2020b). On the other hand, our results did not show any significant effects of glucose injection on the

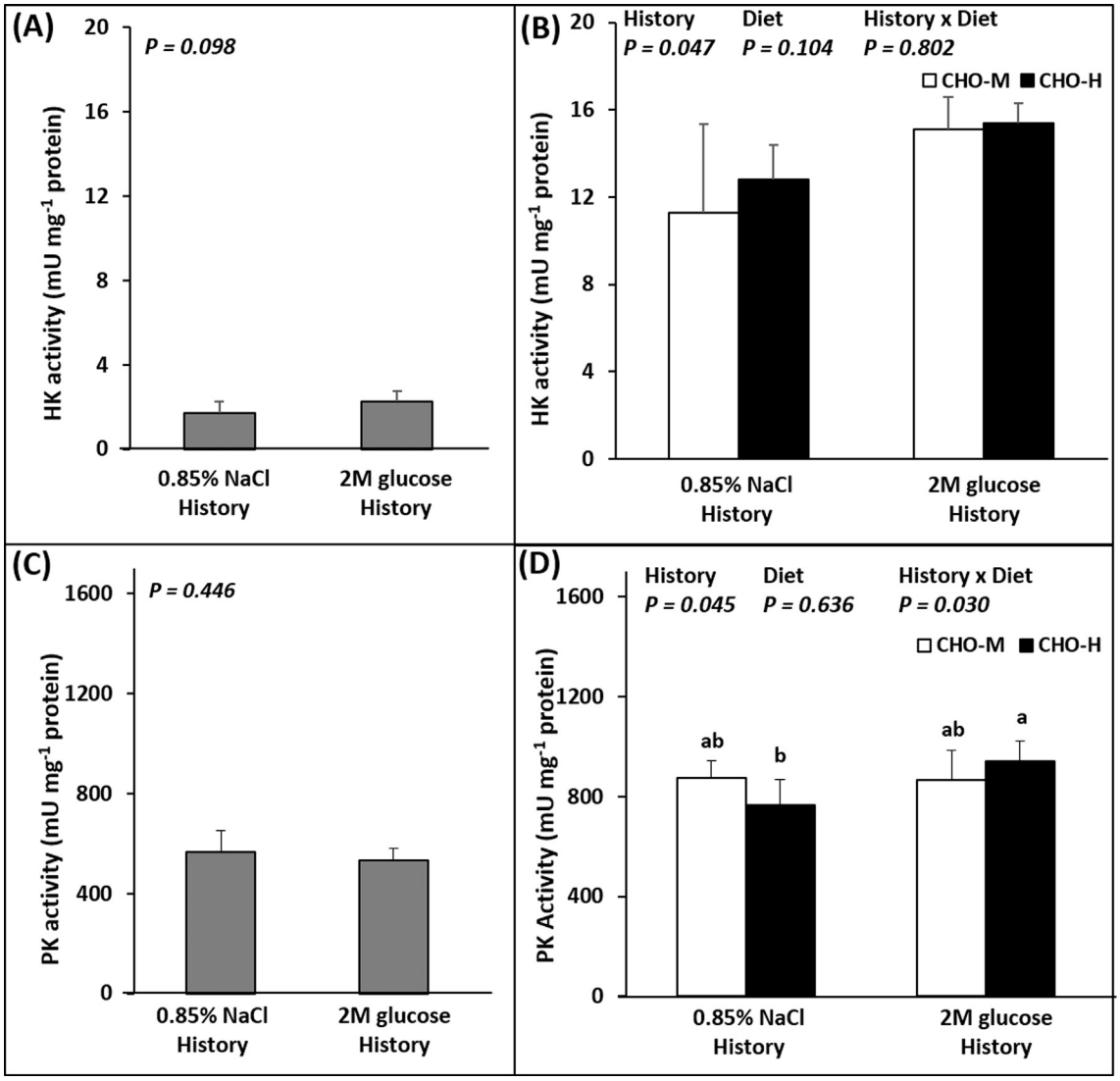

Fig. 3. Muscular enzyme activity (mU mg^{-1} protein) of adult tilapia that were injected with either 0.85% NaCl or 2 M glucose (history). Enzymes involved in hepatic glycolysis included hexokinase (HK) (A, B) and pyruvate kinase (PK) (C, D). At week 32, enzymes were analysed to evaluate the effect of glucose stimuli on enzyme activities (A, C) and the combination effects of glucose injection history and dietary challenging at week 37 (B, D). During weeks 33–37, fish were challenged with different levels of dietary carbohydrate (37% carbohydrates, CHO-M; 67% carbohydrates, CHO-H). Data are presented as the mean ± SD (n = 6). An independent t-test was used to determine the effects of glucose injection before challenging. Two-way ANOVA was performed to analyse the effects of glucose injection (History), challenging diet (Diet) and their interaction (History × Diet). One-way ANOVA followed by Tukey's range test was performed to rank the treatment combination groups when significant interaction effects were observed. Different letters in the bar graph indicate significant differences (P < 0.05).

growth performance in adult fish in contrast to previously observed results in juveniles (Kumkhong et al., 2020a). Undetectable positive effects of early glucose stimulus might be due to life stage associated change in carbohydrate metabolism in fish. Indeed, adult tilapia has higher ability to utilise high dietary carbohydrate as energy than that of juvenile tilapia (FAO, 2021) and so the impact of programming could be undetectable at this developmental stage. Another possibility would be that the programming could be erased after a long period of life. Collectively, the long-term effects of glucose injection in the juvenile stage seem to be absent in the adult stage.

While the effect of the CHO-H diet led to increased glucose and triglyceride and decreased BUN levels in adult tilapia, as previously observed (Boonanuntanasarn et al., 2018a; 2018b), our results showed that early glucose stimulus had no significant effects on plasma metabolite levels in adult tilapia, which is similar to previous results (Kumkhong et al., 2020a). Further investigations regarding intermediary metabolism at the molecular and enzymatic levels are warranted to clarify whether early glucose injection could have permanent effects in the later stages.

Early glucose injection has a permanent effect on metabolism in adult Nile tilapia, particularly when fed with high amounts of carbohydrates

Before the challenge, although there were no significant effects on the composition of chemicals in the liver as well as HSI in adulthood, an increase in muscle glycogen content linked to early glucose injection was noted. These results were similar to findings in the juvenile stage (Kumkhong et al., 2020a). Nevertheless, the effects of early glucose injection were revealed after the challenge. Indeed, glucose stimulus history led to lower protein contents and higher lipid contents in liver and increased glycogen contents in muscle tissue, suggesting that the glucose experience may be associated with modifications in metabolic processes in adult life, as has been previously observed in juvenile tilapia (Kumkhong et al., 2020a). In the present study, as shown previously

(Boonanuntanasarn et al., 2018a; 2018b), fish fed with the CHO-H diet had (1) decreased hepatic and muscle protein levels, (2) increased fat content in the liver, (3) increased hepatic glycogen content and (4) higher liver volume, confirming the effects of dietary carbohydrates on the intermediary metabolism (Polakof et al., 2012; Kamalam et al., 2017). Finally, early glucose injection and high-carbohydrate intake act together to synergistically decrease the protein level in liver and muscle tissue and increase the lipid content and liver volume. These findings strongly suggest the existence of programming due to early glucose injection. It was thus important to check if these effects could be linked to specific mechanisms on intermediary metabolism at the molecular and enzymatic levels.

To determine whether glucose stimulus at the alevin stage had long-term effects on glucose metabolic pathways, we first measured the mRNA levels of several genes involved in hepatic glycolysis, gluconeogenesis, lipogenesis and amino acid catabolism together with muscular glycolysis and glucose transporter prior to dietary challenge. Except for *pfkma*, injection of glucose into the yolk reserve had no significant long-term effects on the expression of glucose metabolism-related genes. The activities of GCK and PK in liver tissues and HK and PK in muscle tissues confirmed that there were no changes. These results are different from those of our previous report on juvenile tilapia by Kumkhong et al. (2020a), which demonstrated that microinjection of glucose into yolk reserves had direct significant effects on up-regulation of hepatic *gck* and muscle *hk1* and *hk2*, as well as on their activities. Again, the effects of early glucose stimulus on metabolism were stronger in juvenile fish than in adulthood. This was supported by the absence of effects of early glucose stimulus on plasma metabolite and chemical composition in liver and muscle. Taken together, our data suggest that the programming effects observed in juveniles (Kumkhong et al., 2020a) seem to be absent in the later stages (adult stage). Because the effects on glucose metabolism could be potentially higher in adult fish fed with high-carbohydrate diets, we therefore analysed the effects of two challenged diets in adult tilapia in subsequent experiment.

For dietary challenging, Nile tilapia were fed with either a medium amount of carbohydrates or high amount of carbohydrates for 5 weeks. In adult tilapia, high dietary carbohydrate ($\geq$12 weeks) influenced the expression of several genes related to carbohydrate metabolism. Indeed, fish fed with carbohydrates showed higher levels of lipogenic *fasn* and *g6pd* mRNAs and muscle glycolysis (*hk2* and *pkma*) as well as lower levels of gluconeogenic *g6pca2*, *pck1*, *pck2* and *asat* (amino acid catabolism), as previously observed (Boonanuntanasarn et al., 2018b). Noticeably, the effects of high dietary carbohydrate challenging in adult stage were less pronounced when compared to that in juvenile fish (Kumkhong et al., 2020a). On the other hand, we found that early glucose injection was associated with the induction of glycolytic enzymes (hepatic *gck* and muscle *hk2*, mRNA and enzymatic activities; and muscle PK activity). Moreover, the hepatic PK activity of adult tilapia injected with glucose is higher but only in fish fed with high amounts of carbohydrates. Our study demonstrated that early glucose injection induced gluconeogenesis, lipogenesis and amino acid catabolism (*asat* and *alat*) in tilapia adults fed with carbohydrates. In addition, we observed the interaction effects between early glucose injection and dietary challenge for the hepatic genes *g6pca2*, *fasn* and *alat* and muscle *glut4*, *pfkma* and *pkma*. Compared with findings of the previous study at the juvenile stage (Kumkhong et al., 2020a), there is a similar metabolic programming in adult tilapia at the molecular and enzymatic levels. Finally, the present findings suggest that early glucose injection modulated carbohydrate metabolism in the long term, with possible induction of glycolysis in the liver and muscles, as well as gluconeogenesis, amino acid catabolism and lipogenesis.

Long-term effects of early glucose injection stimulus are different depending on the life stage

Globally, the long-term effects of early glucose injection on chemical composition in liver and muscle, plasma metabolites and carbohydrate metabolism were similar between juvenile (Kumkhong et al., 2020a) and adult. However, compared to juvenile fish, several effects observed in adult were weaker and some disappeared. The differences in the impacts of nutritional programming between juvenile and adult phases might be due to life stage associated changes in carbohydrate metabolism for energy use in tilapia (FAO, 2021). It might also be due to that the programming effect could be erased after a long period of life because the epigenetic marks at the origin of the programming could be reversible (Langley-Evans, 2009; Orozco-Solís et al., 2010; Gopalakrishnan et al., 2005).

Conclusion

In conclusion, our results showed that early glucose injection during alevin stage had no effect on growth performance in adult fish although glucose injection led to increase muscle glycogen and muscle glycolytic *pfkma* gene expression. The long-term effects of early glucose injection into the yolk on glucose and lipid metabolism were assessed by feeding adult tilapia with a high-carbohydrate diet. However, the nutritional programming was weaker than that found in our previous study on juvenile tilapia. Early glucose stimulus history appeared to promote glycolysis in the liver and muscles, as well as gluconeogenesis, lipogenesis and amino acid catabolism in adult tilapia.

Ethics approval

All experimental protocols were approved by the Ethics Committee of Suranaree University of Technology, Animal Care and Use Committee (approval no. A-18/2562).

Data and model availability statement

None of the data were deposited in an official repository. All the data of this study are available from the corresponding author upon reasonable request.

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E. Plagnes-Juan and V. Veron: Methodology, Formal analysis, validation.
All authors have read and approved the final manuscript.

Declaration of interest

The authors declare that they have no conflict of interest.

Acknowledgements

We would also like to acknowledge the contribution of the French Government (N° de dossier Campus France: 931061D) and the Embassy of France in Thailand to RGJ-Ph.D. program. We would also like to thank Sunai Plymee for maintaining the broodstock.

Financial support statement

This work was supported by Suranaree University of Technology and by the Office of the Higher Education Commission under the NRU Project of Thailand. In addition, financial support from the Thailand Research Fund through the Royal Golden Jubilee Ph. D Program (Grant no. PHD/0138/2557) to student's initials and adviser's initials is acknowledged.

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